The effects of hypothermia on fibrinogen metabolism and coagulation function in swine

Wenjun Zhou Martini

The US Army Institute of Surgical Research, Ft. Sam Houston, TX 78234, USA

Abstract

Clinical coagulopathy frequently occurs in the presence of hypothermia. The primary purpose of this study was to investigate the effects of hypothermia on clotting protein fibrinogen metabolism and on coagulation function in a swine model. Twelve pigs were randomly allocated into control and hypothermia groups. Hypothermia of 32°C was induced using a blanket with circulating water at 4°C. Fibrinogen synthesis and breakdown were quantified using a 6-hour stable isotope infusion with subsequent gas chromatograph and mass spectrometry analysis. Clotting enzyme thrombin generation kinetics was quantified at baseline and at the end of the infusion. Changes in fibrinogen metabolism and thrombin generation were correlated with coagulation function assessed by thromboelastography (TEG). Hypothermia decreased fibrinogen synthesis from the control value of 2.6 ± 0.4 to 1.2 ± 0.2 mg kg^{-1} h^{-1} ($P < .05$), with no effect on fibrinogen breakdown. Thrombin generation at the initiation phase was delayed by hypothermia, but there were no changes at the propagation phase. In thromboelastography measurements, the initial clotting time (R time) was prolonged from the baseline value of 3.01 ± 0.13 to 4.30 ± 0.24 minutes ($P < .05$) and clotting rapidity (angle α) was decreased from the baseline value of 72.30 ± 0.90 to 65.34 ± 1.07 ($P < .05$). Hypothermia caused no significant changes in clot strength (maximum amplitude) and clot lysis (LY$_{60}$). We concluded that hypothermia caused a potential deficit in fibrinogen availability and a delay in thrombin generation, consequently inhibiting coagulation function. Our data support the current practices of rewarming and prescribing recombinant factor VIIa for hypothermic patients with coagulation defects.
Published by Elsevier Inc.

1. Introduction

Hypothermia, clinically defined as body temperature of 34°C or less, is commonly observed in severely injured trauma patients. As much as 66% of trauma patients arrive in emergency departments with temperatures of less than 36°C [1]. Among nonsurviving trauma patients, approximately 80% have body temperatures of less than 34°C at the time of death [2]. One of the most distressing complications of hypothermia is the disruption of hemostasis. Compared with patients with body temperatures of 36.1°C $\pm$ 0.7°C, there was a 2.4-fold increase of blood loss in postlaparotomy patients whose body temperature was 33.8°C $\pm$ 0.5°C [3]. The association of hypothermic coagulopathy with high mortality has been well described [3-9]. In a group of patients with injury severity scores of more than 25, the mortality increased from 10% to 100% when body

temperatures declined from 35°C to less than 32°C [7]. The complexity of clinical settings, such as tissue injury, blood loss, resuscitation, and hypothermia, makes it difficult to clarify the mechanisms related to coagulation defects in hypothermic patients. Therefore, we used a swine model to define the effects of hypothermia on the coagulation process in this study.

The coagulation process involves fibrin clot formation from the precursor fibrinogen. Thrombin, catalyzing the conversion of fibrinogen to fibrin clot, plays an important role in this process [10]. Thrombin is generated from prothrombin during the initiation and propagation phases. In the initiation phase, a small amount of thrombin is produced as a result of the formation of the activated tissue factor and factor VIIa complex. In the propagation phase, a large amount of thrombin is generated upon the activation of a series of procoagulant enzymes (ie, factors V, VIII, and IX) and platelets. Blood hemostasis requires rapid thrombin generation and sufficient fibrinogen in the circulation. The effects of hypothermia on the coagulation process have been estimated

		Form Approved
Report Documentation Page		OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 FEB 2007	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
The effects of hypothermia on fibrinogen metabolism and coagulation function in swine	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Martini W. Z.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234	

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**8**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

by cold-induced changes in standard clinical laboratory tests of prothrombin time (PT) and activated partial thromboplastin time (PTT) [11-16]. Prolonged PT and PTT have been shown in hypothermic patients and experimental animals, as well as plasma samples cooled in vitro [11-16]. Because PT and PTT provide estimates of the extrinsic-common and the intrinsic-common pathways, respectively, those measurements could not reflect the effects of hypothermia on a specific enzyme, such as thrombin. In addition, the effects of hypothermia on dynamic aspects of fibrinogen metabolism have not been identified.

This study was designed to investigate the effects of hypothermia on fibrinogen metabolism, thrombin generation, and coagulation function in vivo. After the induction of hypothermia in a swine model, fibrinogen synthesis and breakdown were quantified using our newly developed stable isotope infusion technique [17], together with quantifications in thrombin generation kinetics. Changes in fibrinogen metabolism and thrombin generation were correlated with coagulation functional assessment by thromboelastography (TEG).

2. Methods

2.1. Experimental design

This study was approved by the US Army Institute of Surgical Research Institutional Animal Care and Use Committee (A-03-003). A total of 12 crossbred commercial juvenile Yorkshire swine (40.9 ± 0.8 kg, John Albert Swine Farm, Cibolo, TX) were randomized into 2 experimental groups: normal control (n = 6) and hypothermia (n = 6). After an overnight fast, animals were preanesthetized with glycopyrrolate (0.1 mg/kg, IM) and Telazol (6 mg/kg, IM; Fort Dodge Animal Health, Fort Dodge, IA). The pigs were then intubated and anesthesia maintained by 1.5% to 2.5% isoflurane in 100% oxygen by mask for the surgical procedures. A multiple sensor (Paratrend Diametrics Medical, High Wycombe, UK) was inserted in the right carotid artery for measuring mean arterial pressure, temperature, pH, and heart rate. A Swan-Ganz thermodilution catheter was inserted in the left jugular vein to measure cardiac output. The right femoral artery was cannulated for blood sampling and the left femoral vein was cannulated for infusions of stable isotopes and maintenance fluid. The right femoral vein was cannulated for intravenous anesthesia of ketamine during the study.

Upon completion of surgical procedures, lactated Ringer's solution was infused at 0.04 mL kg^{-1} min^{-1} as a maintenance fluid in both control and hypothermia groups. Anesthesia was switched to a combination of isoflurane (0.5%) and continuous intravenous drip of ketamine (0.15 mL kg^{-1} h^{-1} of 100 mg/mL) in all pigs for the reminder of the study period. After a 10-minute stabilization period, blood samples were taken from the femoral artery for baseline measurements. Hypothermia was induced in the hypothermia group by means of a water-pumped blanket

with recirculating water at 4°C until the animal's body temperature reached 32.0°C (blankets were not used in the control group). Afterward, pig body temperature was maintained at 32°C ± 0.5°C throughout the remainder of the study. The Paratrend sensor for temperature measurement was placed into the right carotid artery and the recirculated-water blanket (4°C) was placed on the pig (from neck to groin). Because the temperature gradient from the sensor (32°C) to blanket (4°C) remained unchanged during the 6-hour isotope infusion, the likelihood of artificially cooling the sensor by the blanket was minimal. Pig body temperatures in the control group (without blankets) were maintained at 39.0°C ± 0.2°C throughout the study. No heparin was used in this study.

2.2. Stable isotope infusion for fibrinogen metabolism

The effects of hypothermia on fibrinogen metabolism were investigated using our newly developed stable isotope technique [17]. Upon the completion of hypothermia induction, priming doses of stable isotopes containing 1-^{13}C-phenylalanine (18 μmol/kg) and d$_5$-phenylalanine (18 μmol/kg) were given, followed immediately by a constant infusion of tracer 1-^{13}C-phenylalanine (0.3 μmol kg^{-1} min^{-1}) and d$_5$-phenylalanine (0.3 μmol kg^{-1} min^{-1}). The infusion of 1-^{13}C-phenylalanine was maintained for 6 hours and the d$_5$-phenylalanine infusion was maintained for 4 hours (Fig. 1). Blood samples (10 mL each) were

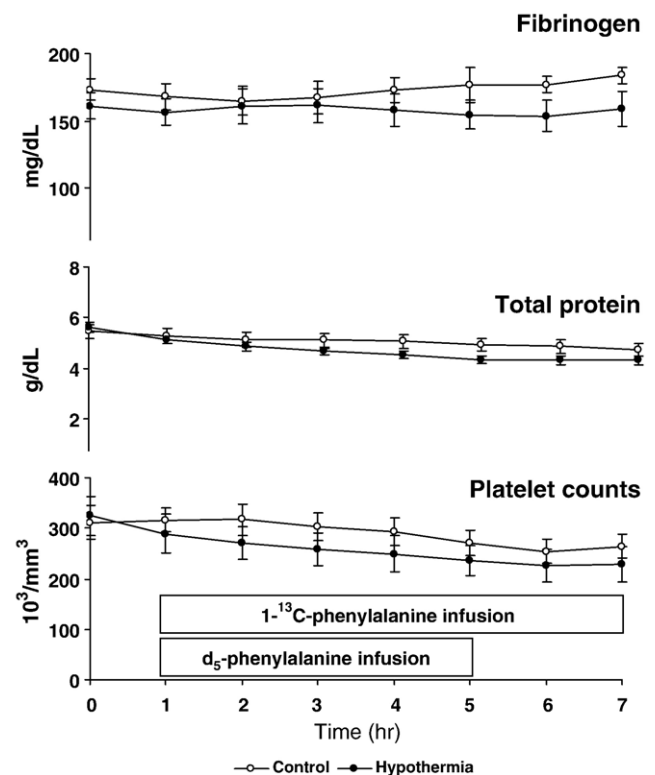Fig. 1. Measurement of total plasma protein and fibrinogen concentrations and blood platelet levels during hypothermia (32°C) in pigs.

collected from the femoral artery cannula at 0, 1, 2, 3, 4, 5, 5.5, 6, 6.5, and 7 hours. Of the 10-mL blood, 3 mL was used for measurements of blood chemistry (green-top vacutainer tube, Fisher, Houston, TX) and platelet (purple-top vacutainer EDTA tubes, Fisher); 2 mL for fibrinogen concentration analysis (blue-top vacutainer tubes, Fisher); and 5 mL for fibrinogen kinetics analysis (purple-top vacutainer EDTA tubes, Fisher). A bolus injection of sterile indocyanine green was given and blood samples (2 mL each, in gold-top vacutainer tubes, Fisher) were collected before the injection and at 5, 10, and 15 minutes postinjection to assess pig plasma volume. Additional blood samples (3 mL each) were collected at 0 and 5 hours to assess changes in coagulation function. Thus, a total of 114-mL blood was collected during the 7-hour study, and this volume of blood was adequately compensated by the volume of maintenance fluid (0.04 mL kg^{-1} min^{-1}). At the end of the isotope infusion, animals were euthanized with sodium pentobarbital (100 mg/kg, IV).

Mean arterial pressure, temperature, and heart rate were recorded continuously during the study. Cardiac output was determined by thermodilution in triplicate at baseline and hourly throughout the remainder of the study.

2.3. Calculations for fibrinogen synthesis and breakdown

Fibrinogen synthesis and breakdown rates were quantified using a primed constant infusion of stable isotope 1-^{13}C-phenylalanine and d$_5$-phenylalanine (Cambridge Isotope Laboratories, Andover, MA). In stable isotope d$_5$-phenylalanine, 5 hydrogen atoms (^{1}H, mass number of 1) in naturally present phenylalanine $C_6H_5CH_2CH(NH_2)COOH$ (FW 165) are replaced by 5 deuterium (^{2}H, mass number of 2) to become $C_6d_5CH_2CH(NH_2)COOH$ (FW 170). Calculations for fibrinogen synthesis and breakdown have been described previously [17]. Briefly, plasma fibrinogen fractional synthesis rate (FSR) was calculated using the formula:

$$FSR = \left(EB_{(t2)} - EB_{(t1)}\right)/\left(EF \times t\right) \quad (1)$$

where $EB_{(t)}$ is the enrichment of fibrinogen-bound amino acids and EF is the enrichment of plasma amino acids (precursor enrichment). Enrichment is defined as tracer (labeled amino acids) to tracee (unlabeled amino acids) ratio (TTR).

Plasma fibrinogen fractional breakdown rate (FBR) was calculated using the formula:

$$FBR = \left(EB_{pred} - EB_{act}\right)/\left(EB_{(t1)} \times t\right) \quad (2)$$

$$EB_{pred} = EB_{(t1)} + FSR \times EF \times t \quad (3)$$

where EB_{pred} is the predicted fibrinogen-bound d$_5$-phenylalanine enrichment, EB_{act} is the actual measured fibrinogen-bound d$_5$-phenylalanine enrichments, $EB_{(t1)}$ is the starting fibrinogen-bound phenylalanine enrichment when d$_5$-phenylalanine infusion is stopped, EF is the enrichment of plasma phenylalanine, and t is the amount of time that has elapsed from the starting fibrinogen-bound phenylalanine

measurement. Specifically in this study, d$_5$-phenylalanine was infused from 1 to 5 hours and stopped at 5 hours. EB_{pre}, calculated using Eq. (3), is the predicted enrichment of fibrinogen-bound amino acid if d$_5$-phenylalanine infusion had not stopped at 5 hours but had continued to the end of the study. EB_{act} was the actual measured fibrinogen phenylalanine enrichment with d$_5$-phenylalanine infusion stopped at 5 hours. The values of EF, EB_{pre}, and EB_{act} varied with time during the infusion study. For example, in one representative pig, EF, EB_{pre}, and EB_{act} were 7.54%, 2.04%, and 1.95%, respectively, at 5 hours, and 5.02%, 2.13%, and 2.06% at 5.5 hours. The changing patterns of these parameters between 5 and 7 hours allowed the calculation of the fibrinogen breakdown rate using Eqs. (2) and (3).

Fibrinogen FBRs represent the fraction (%) of total fibrinogen breakdown (or loss) during a unit of time; absolute breakdown rates represent the amount of fibrinogen (mg) breakdown per kilogram of body weight during a unit of time. For example, FBR of 5%/h means a 5% breakdown of the total fibrinogen pool in 1 hour; ABR of 3 mg kg^{-1} h^{-1} means a 3-mg breakdown of fibrinogen per kilogram of body weight in 1 hour. Absolute breakdown rate was calculated by multiplying FBR times pig total plasma volume and fibrinogen concentration (ABR = FBR × plasma volume × fibrinogen concentration). Similarly, fibrinogen absolute synthesis rate was calculated by multiplying FSR times pig total plasma volume and fibrinogen concentration.

In stable isotope tracer methodology, protein synthesis and breakdown rates are independent of the tracers used for the quantifications. Specifically in this study, fibrinogen incorporation rate calculated from d$_5$-phenylalanine is the same as that from 1-^{13}C-phenylalanine. Likewise, fibrinogen breakdown rate from 1-^{13}C-phenylalanine would have been the same as that from d$_5$-phenylalanine in this study if 1-^{13}C- phenylalanine was infused for 4 hours and stopped at 5 hours. In our calculation, it was assumed that the plasma fibrinogen pool acted like a single pool for calculation of FBR. Previous studies have used a 2-pool model to describe fibrinogen turnover and have concluded that the 2 pools represent intravascular and extravascular spaces [18-21]. However, these studies were conducted over a period of 6 to 15 days and the effect from the extravascular pool on the intravascular pool could only be detected after days [18-21], indicating that it takes more than 1 day for the single-pool assumption to be invalid. Thus, considering the time frame used in this study (7 hours), it is reasonable to assume that the plasma fibrinogen pool acts like a single pool.

2.4. Thrombin generation kinetics

Thrombin generation was assessed by measuring thrombin–antithrombin III (TAT) complex from fresh whole blood samples, following the procedure described by Rand et al [22]. Briefly, thrombin generation analysis was performed in a closed temperature-controlled chamber. Chamber temper-

Table 1
Physiologic changes after hypothermia (32°C) in pigs

	Mean arterial pressure (mm Hg)	Heart rate (bpm)	Cardiac output (L/min)
Control group			
Baseline (0 h)	87 ± 4	107 ± 6	3.6 ± 0.4
End (7 h)	80 ± 5	115 ± 5	3.2 ± 0.3
Hypothermia group			
Baseline (0 h)	85 ± 3	108 ± 5	3.9 ± 0.6
End (7 h)	53 ± 3*	66 ± 14*	1.8 ± 0.2*

Data are expressed as means ± SE.
 * $P < .05$ compared with corresponding baseline values.

atures were set and maintained to match animal body temperature at the time blood samples were withdrawn. Thus, measured thrombin generation reflected that of the animal. Fresh whole blood samples were aliquoted into 12 tubes containing 20 mmol/L HEPES, 150 mmol/L NaCl, and 5 mmol/L CaCl₂. A "quench" solution (50 mmol/L EDTA and 10 mmol/L benzamidine in HEPES-buffered saline) was added to each of the aliquots at different time intervals to stop clot formation. The quenched samples were centrifuged and supernatants were collected for TAT concentration measurement using commercially available ELISA kits (Enzygnost TAT, Dade Behring, Deerfield, IL). The TAT concentrations from the supernatant samples reflect thrombin content generated from fresh whole blood samples before quench time points. This technique has been used to assess thrombin generation kinetics in various studies [22-24].

2.5. Coagulation function

Thromboelastograph is an instrument designed to monitor and analyze the coagulation state of a blood sample [25]. The TEG measures the changes in clot physical strength using a special stationary cylindrical cup, which is oscillated with rotation cycles. When a blood sample is added to the cup, force changes in rotation movements are converted by a mechanical-electrical transducer to an electrical signal, which is recorded by a computer as a clotting curve. The clotting curve provides a coagulation profile from a measured blood sample. For example, reaction time (R time) represents the latency time for initial detectable clot formation. Angle α measures the rapidity of fibrin clot buildup and cross-linking. Maximum amplitude (MA) represents maximum strength or stiffness of the clot, and LY_{60} indicates the percentage of clot lysis at 60 minutes after MA is achieved.

In this study, coagulation function was assessed by TEG (TEG 5000 Hemostasis Analyzer, Hemoscope, Niles, IL) using fresh whole blood samples and pig thromboplastin (extracted from pig brain tissue following procedures described previously from our laboratory, reference [26]) at the core temperatures of the animals at 0 hour and at 5 hours during the infusion. Blood samples (3 mL each) were gently withdrawn to minimize shear-induced platelet activation.

2.6. Analytical methods

Blood chemistry was determined by standard clinical methods (Dimension Clinical Chemistry System, Dade Behring, Newark, DE). Platelet counts were measured using an ABX Pentra 120 Hematology Analyzer (ABX Diagnostics, Irvine, CA). Plasma fibrinogen concentration was determined based on clotting functional changes using the Blood Coagulation System (Dade Behring, Deerfield, IL).

For assessment of plasma free amino acid enrichments from the infusion of 1-^{13}C-phenylalanine and d$_5$-phenylalanine, 0.2 mL plasma was acidified by 0.3 mL 15% sulfosalicylic acid and the acidified plasma supernatant was loaded onto a cation exchange column (AG 50W-X8 resin, 200-400 mesh, H$^+$ form, Bio-Rad Laboratories, Richmond, CA). Amino acids were separated after elution with 6 N ammonium hydroxide. The extracts were dried under speed vacuum and derivatized by N-methyl-N-(tert-butyldimethyl-silyl)-trifluoroacetamide at 100°C for 1 hour. Plasma fibrinogen was isolated by adding 0.5 mol/L CaCl₂ and thrombin to form fibrin clot, following the procedure described by Stein et al [27]. The purity of fibrinogen by using this procedure has been validated previously by affinity chromatography [28] and by polyacrylamide gel electrophoresis [29]. The clot was then washed and hydrolyzed in 6 N HCl at 110°C for 24 hours and dried under speed vacuum. The released amino acids after hydrolysis were isolated, dried, and derivatized in the same manner as for plasma free amino acids. The enrichments of phenylalanine from plasma free amino acid pool and in fibrinogen protein were determined by gas chromatography-mass spectrometry (model 5973, Hewlett-Packard, Palo Alto, CA) in the electron impact ionization mode. A selective ion-monitoring method was used at nominal mass-to-charge ratio (m/z) of 336 ($m+0$), 337 ($m+1$), 338 ($m+2$), 339 ($m+3$), 340 ($m+4$), and 341($m+5$) for phenylalanine as previously described [17].

2.7. Statistical analysis

All results are expressed as means ± SE. Comparisons between the control and hypothermia groups in fibrinogen synthesis and breakdown measurements were made with Student t test. Comparisons over time in substrate concentrations, thrombin generation, and coagulation parameters
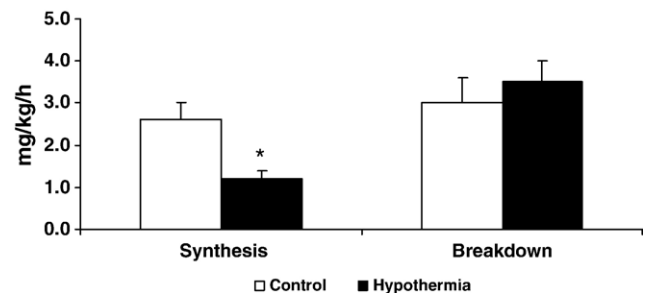

Fig. 2. Effects of hypothermia (32°C) on fibrinogen synthesis and breakdown in pigs. *$P < .05$ compared with control values.
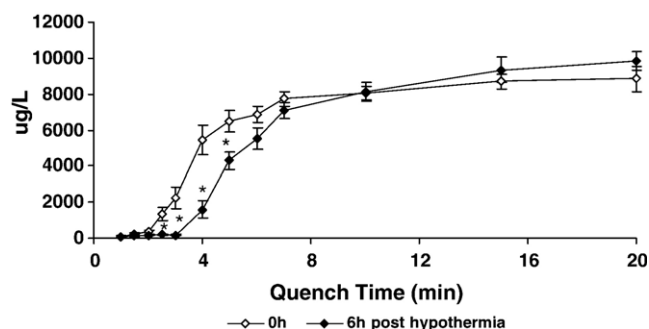
Fig. 3. Changes in thrombin generation kinetics in hypothermic (32°C) pigs. *$P < .05$ compared with corresponding quench time values from baseline samples in the same group.

were made using Bonferroni multiple comparisons test relative to baseline. Statistical significance was set at the .05 level.

3. Results

3.1. Hemodynamics and substrate concentration

Hypothermia was successfully induced in about 1 hour in the hypothermia group. All 12 pigs survived the 6-hour isotope infusion study. There were no differences in all baseline measurements between the 2 groups. There were also no significant changes in the hemodynamics in the control group over the entire experimental period. Upon the completion of hypothermia induction, there were significant decreases in mean arterial pressure, heart rate, and cardiac output (Table 1). These measurements remained unchanged during the remainder of the study.

At baseline (0 hour), plasma fibrinogen concentration was similar in the control (173 ± 9 mg/dL) and hypothermia (161 ± 13 mg/dL) groups. Plasma total protein was also similar in the control (5.5 ± 0.3 g/dL) and the hypothermia (5.6 ± 0.2 g/dL) groups. Platelet counts at baseline were $311 \pm 34 \ 10^3/\mu L$ in the control group and $325 \pm 39 \ 10^3/\mu L$ in the hypothermia group. During the 6-hour isotope infusion, there were no significant changes in fibrinogen concentration, total protein content, or platelet count in either group (Fig. 1). Plasma volume, measured by indocyanine green dye at 5 hours during the infusion, was 50.0 ± 0.5 mL/kg in the control group and 55.4 ± 2.0 mL/kg in the hypothermia group ($P < .05$).

3.2. Fibrinogen synthesis and breakdown

The enrichment of plasma free phenylalanine TTR ($m+1$) reached plateau values in both animal groups after 1-hour infusion of 1-^{13}C-phenylalanine ($21.23\% \pm 0.58\%$ in the control group and $25.68\% \pm 1.03\%$ in the hypothermia group, $P < .05$). Plasma fibrinogen-bound phenylalanine enrichment TTR ($m+1$) increased linearly during the infusion of 1-^{13}C-phenylalanine. Fibrinogen FSRs, calculated from the increased slope of fibrinogen-bound phenylalanine enrichment ($m+1$), was $2.7\% \pm 0.2\%$/h in the control group

and $1.4\% \pm 0.1\%$/h in the hypothermia group ($P < .05$). The absolute synthesis rate, calculated by multiplying FSR with plasma volume and fibrinogen concentration, was decreased from the control value of 2.6 ± 0.4 to 1.2 ± 0.2 mg kg^{-1} h^{-1} by hypothermia ($P < .05$, Fig. 2).

Fibrinogen FBRs were calculated based on the changes of fibrinogen phenylalanine enrichment TTR ($m+5$) after the cessation of tracer d$_5$-phenylalanine infusion. The calculated FBRs were $3.6\% \pm 1.0\%$/h in the control group and $4.0 \pm 0.6\%$/h in the hypothermia group ($P > .05$). The ABRs, calculated by multiplying FBR with plasma volume and fibrinogen concentration, were not significantly different between the hypothermia and control groups (Fig. 2).

3.3. Thrombin generation kinetics

Thrombin generation was measured at baseline and at the end of the study. There were no differences in thrombin generation kinetics at baseline (0 hour) between the 2 groups. In the control group, there were no changes in thrombin generation from baseline to the end of the study. However, there was a delay in the initiation phase of thrombin generation by hypothermia. At the 3-, 4-, and 5-minute quench time points from the samples taken at the end of the study, thrombin generation in the hypothermia group decreased to $11\% \pm 3\%$, $30\% \pm 9\%$, and $65\% \pm 14\%$ of the corresponding baseline values from the same group (all $P < .05$, Fig. 3). Beyond the 5-minute quench time, there were no significant differences in thrombin generation between the samples taken at baseline (0 hour) and at the end of the study (Fig. 3). Thus, hypothermia primarily inhibited the initiation phase of thrombin generation.
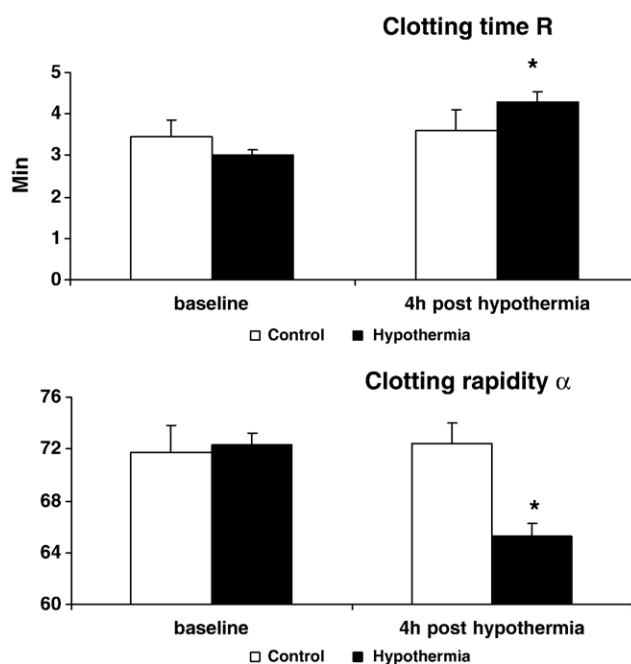

Fig. 4. Effects of hypothermia (32°C) on coagulation function by TEG in pigs. *$P < .05$ compared with baseline values in the same group.

3.4. Coagulation function

Coagulation was measured at 0 hour and at 5 hours during the infusion. There were no changes in TEG measurements in the control group during the study. In the hypothermia group, the initial clotting time (R time) was prolonged from the baseline value of 3.01 ± 0.13 to 4.30 ± 0.24 minutes ($P < .05$, Fig. 4). Clotting rapidity (angle α) was decreased from the baseline value of 72.30 ± 0.90 to 65.34 ± 1.07 ($P < .05$, Fig. 4). There were no significant changes in clot strength (MA) and fibrinolysis (LY_{60}) by hypothermia (data not shown).

4. Discussion

Sufficient fibrinogen availability is essential for effective clotting. Using our newly established stable isotope technique [17], we investigated hypothermia effects on fibrinogen metabolism and coagulation function. Our data showed, for the first time, that hypothermia of $32°C$ decreased fibrinogen synthesis by about 50% with no changes in fibrinogen breakdown, indicating a potential deficit in fibrinogen availability. This deficit may become more problematic when massive blood loss and resuscitation are present because fibrinogen levels may already be near or below the threshold for normal coagulation function before hypothermia fully develops. In addition, because fibrinogen synthesis under normal circumstances is very slow relative to its pool size (about 3%/h) [17,30], it may take a long time to reflect synthesis changes in fibrinogen concentration. For example, when there is a 50% inhibition in synthesis and no change in breakdown, it would take more than 6 hours to result in detectable changes in fibrinogen concentration (about 10%). Thus, the severe inhibition imposed by hypothermia on fibrinogen synthesis is likely to be masked due to the lack of immediate changes in fibrinogen concentration. Therefore, our metabolic results may support the current practice of immediate rewarming as well as the prescription of blood products to replenish coagulation factors in hypothermic trauma patients.

Our observation of inhibited fibrinogen synthesis without significant changes in fibrinogen breakdown indicates that fibrinogen synthesis and degradation are independently regulated in response to cooling. The significant decreases in fibrinogen synthesis shown in this study were expected because protein synthesis requires a series of signals for gene expression activation as well as translation activation, whereas protein breakdown only requires the presence of proteases. Thus, temperature is likely to have more impact on fibrinogen synthesis than on fibrinogen breakdown. Interestingly, we observed the opposite in pigs with hemorrhagic shock [17]; after a moderate hemorrhage, fibrinogen degradation was accelerated but synthesis remained unchanged in pigs [17]. Apparently, complex mechanisms may be involved in regulating fibrinogen synthesis and degradation. This complexity may be further amplified in clinical settings when the multiple factors of hypothermia, hemorrhage, resuscitation, and tissue injury are all involved. Additional efforts are needed to define the individual and combined effects of these factors on coagulation defects. Nonetheless, our kinetic results from this study demonstrate that there is a potential deficit in fibrinogen availability from hypothermia alone due to the consequences of inhibited production without changes in utilization.

Thrombin, catalyzing fibrinogen to fibrin clot, plays an important role in maintaining hemostasis. In this study, hypothermia of $32°C$ significantly delayed the initiation phase in thrombin generation, but had minimal effects on the propagation phase. Because the initial thrombin is generated upon the formation of tissue factor and factor VIIa complex, our data led us to speculate that the observed inhibition in thrombin generation by hypothermia is primarily in the formation (or activation) of tissue factor and factor VIIa complex. Additional studies are needed to confirm or deny this speculation because we did not measure plasma levels of tissue factor or factor VIIa in this study. Interestingly, we observed a reverse sequence in the effects of acidosis on thrombin generation [31]. In pigs with acidosis of pH 7.1, we found that thrombin generation in the propagation phase was inhibited by acidosis by as much as 50%, with no changes in the initiation phase [31]. Thus, hypothermia and acidosis inhibit thrombin generation kinetics via different mechanisms. Although the mechanisms underlying these differential changes remain to be elucidated, it appears that different approaches should be used to recover thrombin activities in hypothermic and acidotic coagulopathy.

Using cell-based in vitro clotting assays, Wolberg et al [32] and Meng et al [33] investigated the systemic effects of hypothermia on enzyme activities, thrombin generation, and coagulation function. The authors reported that there were no significant changes in tissue factor activities on phospholipids and monocytes when temperature dropped from $37°C$ to $33°C$, but significant inhibitions occurred when temperature dropped to less than $33°C$ [31,32]. Thrombin generation decreased by 5% and 64% of the value at $37°C$ when temperature dropped from $37°C$ to $33°C$ and $23°C$, respectively [32]. Similarly, we observed inhibitions in thrombin generation by hypothermia in our study, using an in vivo model and a different thrombin generation assay. Furthermore, the inhibition appears to be likely in the initiation phase (during the formation of factor VII and tissue factor complex). Thus, findings from references [32] and [33] and the present study support the notion that recombinant factor VIIa is a potential therapy for reversing hypothermia coagulopathy.

Our measurements of fibrinogen metabolism and thrombin generation correlated well with coagulation functional assessment by TEG. Reaction time (R) in TEG is the latency time for initial detectable clot formation. A prolonged R time represents a deficiency in enzyme activity or clotting factor

levels. Angle α measures the rapidity of fibrin buildup and cross-linking. It is affected by the availability of fibrinogen and platelets, but mostly by thrombin activity (amount). In this study, we observed a delayed onset of thrombin generation, which was consistent with a prolonged R time and a decreased α in TEG measurements. Because fibrinogen synthesis rate is very low compared with total fibrinogen pool size, the inhibition in fibrinogen synthesis by hypothermia was not reflected in the change of fibrinogen levels within the short time frame of this study (6-hour hypothermia). Even so, we observed coagulation changes of delayed thrombin generation, prolonged clotting time, and decreased clotting rapidity. Thus, if hypothermia is prolonged, depleted fibrinogen levels would be present and the observed coagulation changes would likely be amplified.

In this study, fibrinogen synthesis rate is the amount of fibrinogen (expressed as milligram per kilogram of body weight) that is synthesized and released to circulation in 1 hour. Fibrinogen breakdown rate is the total loss of fibrinogen (expressed as milligram per kilogram of body weight) from plasma, which includes removal from plasma by cells and loss to the extravascular space. Because our method does not allow the quantification of fibrinogen breakdown from a specific pathway, we were not able to differentiate the effects of hypothermia on fibrinogen breakdown by cell removal, or loss to the extravascular space. Recently, Kamler et al [34] reported that edema formation by the peripheral vascular system of rats was markedly increased during hypothermia, indicating that the loss of plasma protein (including fibrinogen) to the extravascular space might be increased by hypothermia. Additional studies are needed to clarify the effects of hypothermia on individual pathways involved in fibrinogen breakdown.

Normal coagulation process requires a dynamic balance of complex regulations in procoagulant, anticoagulant, and fibrinolytic processes. Together with procoagulant enzymes, platelets and fibrinolysis play important roles in blood coagulation. In this study, we did not observe changes in TEG LY_{60} (fibrinolysis). Similar findings have been reported by Watts et al [16] in hypothermic trauma patients and by Kurrek [12] in hypothermic pigs. We also did not observe changes in platelet counts, which was consistent with unchanged MA in TEG measurements. Similarly, Staab et al [15] reported that temperature changes in minipigs did not cause changes in platelet aggregations. However, Watts et al [16] showed significant alterations in platelet function by TEG in hypothermic patients. Although species differences and trauma injury might contribute to the discrepancy, additional investigation is required to clarify the effects of hypothermia on platelet function.

A swine model was used in this study to investigate the effects of hypothermia on fibrinogen metabolism and coagulation. The value of fibrinogen synthesis in our control pigs (2.6 ± 0.4 mg kg^{-1} h^{-1}) was very close to that of reported values (3 mg kg^{-1} h^{-1}) in normal volunteers [35]. To our knowledge, data from direct quantification of fibrinogen degradation in normal humans are not available at present. Thus, we are not able to compare degradation rates between pigs and humans. However, the values between synthesis and degradation in our control pigs indicate that pigs are a reasonable model for fibrinogen metabolic studies. With respect to coagulation, the pig has become the established animal model due to the similarities in human and porcine coagulation processes [36,37]. Thromboelastography has been used to assess blood coagulation profiles for several decades. Its high sensitivity in detecting and monitoring coagulation changes has been recognized in clinical studies since the 1970s [38] Comparable values of clotting formation time, MA, and clotting rapidity have been reported by Velik-Salchner et al [39] in blood samples from human and pigs. However, Landskroner et al [40] recently showed a difference in ex vivo lysis assays between human and pig plasma samples, indicating possible differences in fibrinolysis between human and pigs. Although the validity remains to be clarified, a swine model appears to be a reasonable choice for coagulation studies at this time.

In summary, we investigated the effects of hypothermia on the coagulation process in a swine model. Differential effects of hypothermia were observed on fibrinogen synthesis and degradation, as well as on the initiation and propagation phases in thrombin generation. Hypothermia inhibited fibrinogen production, delayed initial thrombin generation, and consequently compromised the clotting process. Based on our results, rewarming is highly recommended due to the potential deficit in fibrinogen availability and thrombin generation inhibition caused by hypothermia. In addition, recombinant factor VIIa may be a beneficial adjunct in hypothermic patients with bleeding due to its potential to release the inhibition in the initiation phase of thrombin generation.

Acknowledgments

References

[1] Luna GK, Maier RV, Pavlin EG, et al. Incidence and effect of hypothermia in seriously injured patients. J Trauma 1987;27:1014-8.

[2] Ferrara A, MacArthur JD, Wright HK, et al. Hypothermia and acidosis worsen coagulopathy in the patient requiring massive transfusion. Am J Surg 1990;160:515-8.

[3] Bernabei AF, Levison MA, Bender JS. The effects of hypothermia and injury severity on blood loss during trauma laparotomy. J Trauma 1992;33:835-9.

[4] Britt LD, Dascombe WH, Rodriguez A. New horizons in management of hypothermia and frostbite injury. Surg Clin North Am 1991;71: 345-70.

[5] Gentilello LM, Cortes V, Moujaes S, et al. Continuous arteriovenous rewarming: experimental results and thermodynamic model simulation of treatment for hypothermia. J Trauma 1990;30:1436-49.

[6] Gregory JS, Flancbaum L, Townsend MC, et al. Incidence and timing of hypothermia in trauma patients undergoing operations. J Trauma 1991;31:795-8 [discussion 798-800].

[7] Jurkovich GJ, Greiser WB, Luterman A, et al. Hypothermia in trauma victims: an ominous predictor of survival. J Trauma 1987;27: 1019-24.

[8] Prakash O, Jonson B, Bos E, et al. Cardiorespiratory and metabolic effects of profound hypothermia. Crit Care Med 1978;6:340-6.

[9] Steinemann S, Shackford SR, Davis JW. Implications of admission hypothermia in trauma patients. J Trauma 1990;30:200-2.

[10] Mann KG, Brummel K, Butenas S. What is all that thrombin for? J Thromb Haemost 2003;1:1504-14.

[11] Kermode JC, Zheng Q, Milner EP. Marked temperature dependence of the platelet calcium signal induced by human von Willebrand factor. Blood 1999;94:199-207.

[12] Kurrek MRR. Effect of hypothermia on enzymatic activity of thrombin and plasmin. Surg Forum 1987;38:221-3.

[13] Reed II RL, Johnson TD, Hudson JD, et al. The disparity between hypothermic coagulopathy and clotting studies. J Trauma 1992;33: 465-70.

[14] Rohrer MJ, Natale AM. Effect of hypothermia on the coagulation cascade. Crit Care Med 1992;20:1402-5.

[15] Staab DB, Sorensen VJ, Fath JJ, et al. Coagulation defects resulting from ambient temperature-induced hypothermia. J Trauma 1994; 36:634-8.

[16] Watts DD, Trask A, Soeken K, et al. Hypothermic coagulopathy in trauma: effect of varying levels of hypothermia on enzyme speed, platelet function, and fibrinolytic activity. J Trauma 1998; 44:846-54.

[17] Martini WZ, Chinkes DL, Pusateri AE, et al. Acute changes in fibrinogen metabolism and coagulation after hemorrhage in pigs. Am J Physiol Endocrinol Metab 2005;289:E930-4.

[18] Blaisdell FW, Graziano CJ. Assessment of clotting by the determination of fibrinogen catabolism. Am J Surg 1978;135:436-42.

[19] Blaisdell FW, Graziano CJ, Effeney DJ. In vivo assessment of anticoagulation. Surgery 1977;82:827-39.

[20] Tytgat GN, Collen D, Vermylen J. Metabolism and distribution of fibrinogen. II. Fibrinogen turnover in polycythaemia, thrombocytosis, haemophilia A, congenital afibrinogenaemia and during streptokinase therapy. Br J Haematol 1972;22:701-17.

[21] Tytgat GN, Collen D, Verstraete M. Metabolism of fibrinogen in cirrhosis of the liver. J Clin Invest 1971;50:1690-701.

[22] Rand MD, Lock JB, van't Veer C, et al. Blood clotting in minimally altered whole blood. Blood 1996;88:3432-45.

[23] Butenas S, Brummel KE, Branda RF, et al. Mechanism of factor VIIa–dependent coagulation in hemophilia blood. Blood 2002;99:923-30.

[24] Butenas S, Brummel KE, Bouchard BA, et al. How factor VIIa works in hemophilia. J Thromb Haemost 2003;1:1158-60.

[25] Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis 2001;12:327-37.

[26] Pusateri AE, Ryan KL, Delgado AV, Martinez RS, Uscilowicz JM, Cortez DS, et al. Effects of increasing does of activated recombinant factor VII on haemostatic parameters in swine. Thromb Haemost 2005;93:275-83.

[27] Stein TP, Leskiw MJ, Wallace HW. Measurement of half-life human plasma fibrinogen. Am J Physiol 1978;234:D504-10.

[28] Heene DL, Matthias FR. Adsorption of fibrinogen derivatives on insolubilized fibrinogen and fibrin monomer. Thromb Res 1973;2: 137-54.

[29] Mansoor O, Cayol M, Gachon P, et al. Albumin and fibrinogen syntheses increase while muscle protein synthesis decreases in head-injured patients. Am J Physiol 1997;273:E898-E902.

[30] Jahoor F, Wykes L, Del Rosario M, et al. Chronic protein undernutrition and an acute inflammatory stimulus elicit different protein kinetic responses in plasma but not in muscle of piglets. J Nutr 1999;129:693-9.

[31] Martini WZ, Pusateri AE, Uscilowicz JM, et al. Independent contributions of hypothermia and acidosis to coagulopathy in swine. J Trauma 2005;58:1002-10.

[32] Wolberg AS, Meng ZH, Monroe DM, et al. A systemic evaluation of the effect of temperature on coagulation enzyme activity and platelet function. J Trauma 2004;56:1221-8.

[33] Meng ZH, Wolberg AS, Monroe DM, et al. The effect of temperature and pH on the activity of factor VIIa: implication for the efficacy of high-dose factor VIIa in hypothermic and acidotic patients. J Trauma 2003;55:886-91.

[34] Kamler M, Goedeke J, Pizanis N, et al. In vivo effects of hypothermia on the microcirculation during extracorporeal circulation. Eur J Cardiothorac Surg 2005;28:259-65.

[35] Giordano M, De Feo P, Lucidi P, dePascale E, Giordano G, Infantone L, et al. Increased albumin and fibrinogen synthesis in hemodialysis patients with normal nutritional status. J Am Soc Nephrol 2001;12: 349-54.

[36] Olsen AK. The pig as a model in blood and coagulation and fibrinolysis research. Scand J Lab Anim Sci 1999;26:214-24.

[37] Munster AM, Olsen AK, Bladbjerg EM. Usefulness of human coagulation and fibrinolysis assays in domestic pigs. Comp Med 2002;52:39-43.

[38] Lee BY, Trainor FS, Kavner D, et al. Monitoring of heparin therapy with thromboelastography. Surg Gynecol Obstet 1979;149:843-6.

[39] Velik-Salchner C, Schnurer C, Fries D, et al. Normal values for thrombelastography (ROTEM) and selected coagulation parameters in porcine blood. Thromb Res 2006;117:597-602.

[40] Landskroner K, Olson N, Jesmok G. Cross-species pharmacologic evaluation of plasmin as a direct-acting thrombolytic agent: ex vivo evaluation for large animal model development. J Vasc Interv Radiol 2005;16:369-77.